Introduction

Autophagy is defined as an intracellular mechanism for the turnover of cytosolic material dependent on a lytic compartment, that is, a lysosome or the vacuole [1]. It is conserved in eukaryotic species from simple single-cellular fungi like *Saccharomyces cerevisiae* to complex organisms of the Animalia kingdom. Autophagy is capable of rapidly turning over large amounts of cytosolic material and organelles, ensuring cellular homeostasis and cell survival under starvation conditions. There are several different types of autophagy pathways, including macroautophagy, microautophagy and—in mammals—chaperone-mediated autophagy (CMA). They share as a common feature the delivery of cargo material to and its degradation in the vacuole or a lysosome, but differ in the way by which the cargo is delivered to the lytic compartment (Fig. 1).

In macroautophagy, cytosolic material is delivered to the lytic compartment in double-membrane vesicles termed autophagosomes. In *S. cerevisiae*, autophagosomes are formed at a single, perivacuolar site termed pre-autophagosomal structure or phagophore assembly site (PAS). The PAS is defined by the co-localization of the core macroautophagy machinery, which is visible as a dot at the vacuole in fluorescence microscopy [2,3]. Autophagosome formation occurs in distinct steps. At the PAS, vesicles nucleate a double-sheeted isolation membrane. The isolation membrane expands in a cup-shaped fashion around autophagic cargo before closing to form a mature autophagosome. The outer membrane of the autophagosome then fuses with the vacuolar membrane, releasing the inner vesicle into the vacuolar lumen, where its contents are degraded (reviewed in Ref. [4]).

These processes and many of the involved proteins are conserved in other eukaryotes, including mammals. One notable difference in mammals is that isolation membranes are formed not only at one site in each cell but at several sites in parallel [5]. This is likely due to the large size of mammalian cells compared to yeast and the availability of numerous lysosomes as target compartments distributed in the cell. Macroautophagy exists in selective and non-selective forms [6]. In non-selective, "bulk" macroautophagy, a random part of the cytosol is engulfed by the isolation membrane and degraded in the vacuole. This general degradation pathway is induced in starved cells to mobilize resources and energy bound in potentially...
superfluous proteins and organelles. Selective macroautophagy targets surplus or damaged organelles and intracellular structures, such as mitochondria [7], peroxisomes [8], the endoplasmic reticulum [9] and ribosomes [10], limits the activity of retrotransposons [11] and removes ubiquitylated protein aggregates, which cannot be dealt with by proteasomal degradation [12,13]. In S. cerevisiae, the selective macroautophagy machinery delivers several enzymes, including aminopeptidase 1 (Ape1), to the vacuole via the cytosol-to-vacuole targeting (Cvt) pathway [14–16]. In mammalian cells, selective macroautophagy also targets intracellular pathogens [17,18] and ferritin [19–21]. In all these pathways, selectivity is conferred by specialized cargo receptor proteins, which link their specific cargo to the autophagy machinery [6].

In microautophagy, the lytic compartment directly engulfs cytosolic material with its own membrane. The lysosomal or vacuolar membrane invaginates, engulfs the cargo and buds off into the lumen as a vesicle, which is degraded together with its content. In CMA, an HSP7C chaperone complex recognizes target proteins by a KFERQ-like sequence and mediates their transport to the lysosomal membrane. There, the protein is unfolded and translocated into the lysosome through a protein pore by the concerted action of cytosolic and lysosomal HSP7C.

**Fig. 1.** Autophagy pathways. Different autophagy pathways deliver cargo to the lytic compartment by different means. In macroautophagy, a cup-shaped isolation membrane is formed de novo, engulfing cytosolic material and closing in order to form a double-membrane autophagosome. The outer membrane of the autophagosome fuses with the lysosomal or vacuolar membrane and releases the inner vesicle into the lumen, where it is degraded. In microautophagy, cargo is taken up directly by the lytic compartment. The lysosomal or vacuolar membrane invaginates, engulfs the cargo and buds off into the lumen as a vesicle, which is degraded together with its content. In CMA, an HSP7C chaperone complex recognizes target proteins by a KFERQ-like sequence and mediates their transport to the lysosomal membrane. There, the protein is unfolded and translocated into the lysosome through a protein pore by the concerted action of cytosolic and lysosomal HSP7C.
Regulation of autophagy by Atg1/ULK1 signaling

The Atg1/ULK1 Kinase Core Complex

Atg1 is a conserved serine/threonine kinase [31] and, to date, the only kinase specific for autophagy described in S. cerevisiae. Its deletion, or expression of a kinase-dead mutant, leads to the accumulation of most other members of the autophagy machinery at the PAS [3], and to the abrogation of autophagy progression at an early stage [31,32]. Thus, Atg1 is considered as pivotal to the regulation of autophagy. The Atg1 core complex consists of Atg1, Atg13, Atg17–Atg29–Atg31 and Atg11 (Fig. 2a and b). Atg13 is required for Atg1 kinase activity and autophagy progression in both nutrient-rich and starvation conditions [33]. Atg13 is strongly phosphorylated in nutrient-rich conditions, but becomes rapidly dephosphorylated upon nutrient deprivation or inhibition of target of rapamycin complex 1 (TORC1) by treatment with rapamycin [33]. This coincides with increased Atg1 kinase activity and the induction of bulk autophagy [33,34]. In an early study, dephosphorylation of Atg13 was reported to be required for its interaction with Atg1, leading to a model in which Atg13 binds to Atg1 only in starvation conditions [33]. This model has since been revised, as Atg1 function requires Atg13 binding also in fed cells [35], and only the binding affinity is regulated by nutrient signaling [36]. The finding that increased binding of Atg13 to Atg1 coincides with the upregulation of Atg1 kinase activity has led to the assumption that Atg13—under the control of TORC1—acts as a direct activator of Atg1 kinase activity and bulk autophagy, possibly by promoting self-activation of Atg1 in trans [37]. In agreement with this, the phosphorylation of several sites on Atg1 is upregulated during bulk macroautophagy induction. Two of these sites, Thr226 and Ser230, are located in the kinase activation loop and required for kinase function [38,39]. The kinase activity of Atg1 directly regulates isolation membrane expansion, as different Atg1 mutants have been shown to form isolation membranes of different sizes, proportional to their kinase activity [32]. Starvation and TORC1 inhibition also allow for efficient binding of Atg13 to Atg17, another member of the core autophagy machinery. Atg17 is required for PAS organization and Atg1 kinase activity in starvation-induced autophagy [3,33]. It forms a stable sub-complex with Atg29 and Atg31, which are required for proper Atg17 localization and function [40–42]. Atg17 has been reported to form homodimers with an S-shaped structure, reminiscent of two BAR (Bin-Amphiphysin-Rvs) domain dimers binding to each other [42]. BAR domains directly bind curved membranes [43], leading to the speculation that Atg17, together with Atg1, might be involved in binding Atg9-positive vesicles at the PAS [42]. Atg17 has recently been reported to bind to Atg9 directly and to tether Atg9 proteoliposomes in vitro [44]. However, the nature of this interaction is controversial, as the N-terminal Hop1, Rev7 and Mad2 (HORMA) domain of Atg13, but not Atg17, has been found to bind Atg9 in vivo [45]. In selective autophagy, Atg17 is dispensable and the PAS is organized by the scaffold protein Atg11. Atg11 binds to, among other proteins, Atg1, Atg9, Atg29 and the Cvt cargo receptor Atg19, and recruits the autophagy machinery to the autophagic cargo [33,46–49].

The mammalian homologues of Atg1 are the uncoordinated-51-like kinases 1 and 2 (ULK1 and ULK2). ULK1 and ULK2 likely can act redundantly, as ULK1 deficiency causes only a mild phenotype in mice [50]. Their respective importance for autophagy, however, seems to vary depending on the cell line, as silencing of ULK1 alone is sufficient to inhibit autophagy in HEK293 cells [51]. As Atg1, ULK1 harbors an autophosphorylation site, Thr180, in the kinase activation loop [52]. ULK1 is part of a multimeric protein complex, composed of mammalian Atg13, RB1CC1/FIP200 (RB1-inducible coiled-coil protein 1, in autophagy research commonly referred to as FAK family kinase-interacting protein of 200 kDa) and Atg101 (Fig. 2a). In contrast to yeast, the binding affinities within mammalian ULK1 complex seem not to be regulated by nutrient signaling [53–57]. However, subtle differences in affinity might have been masked by protein overexpression in these studies. Small affinity changes might also have been missed because they seemed insignificant compared to the predominant model of yeast autophagy at the time, in which Atg13 was thought not to interact with Atg1 in nutrient-rich conditions. This holds true for autophagy in Drosophila melanogaster, where the interaction of Atg1 and Atg13 was found to increase approximately fourfold upon starvation [58]. Nevertheless, it was reported to not be regulated by nutrient signaling, even though this affinity difference closely resembles the one recently found in yeast [36]. Mammalian Atg13 was independently identified and characterized by several groups in parallel [54,56,57,59]. As yeast Atg13, it features an N-terminal HORMA domain (Fig. 2b), but...
possesses only weak sequence homology to the yeast protein [60]. Mammalian Atg13 is required for targeting the ULK1 complex to sites of autophagosome formation, and for ULK1 stability and kinase activity. Similar to yeast Atg13, mammalian Atg13 is strongly phosphorylated under nutrient-rich conditions, but is only partially

Fig. 2. (legend on next page).
Regulation of autophagy by Atg1/ULK1 signaling

Regulation of Autophagy by Phosphorylation of the Atg1/ULK1 Complex

Autophagy activity is finely tuned to ensure cellular homeostasis. Belated induction of autophagy in response to cellular stress like starvation may be lethal to the cell. At the same time, failure to properly limit autophagy levels may lead to unchecked, detrimental self-digestion. The Atg1/ULK1 complex is pivotal in the induction of autophagy and thus a prime target of regulatory pathways. Indeed, several signaling kinases have been found to phosphorylate Atg1 and ULK1, and their complex partners, and will be discussed in the following sections.

Regulation by TORC1

Yeast Tor1 and Tor2 are conserved serine/threonine kinases that act as major controllers of cell growth and metabolism. Tor kinases form two complexes of different composition, TORC1 (Tor complex 1, containing either Tor1 or Tor2) and TORC2 (Tor complex 2, exclusively containing Tor2). TORC1 activity is regulated by nutrient cues and can be inhibited by rapamycin treatment, whereas TORC2 is resistant to rapamycin treatment and its regulation is not well understood [73,74]. TORC1, but not TORC2, is also a key repressor of autophagy under nutrient-rich conditions, and...
inhibition of TORC1 leads to the rapid induction of autophagy [34]. In S. cerevisiae, Atg1 complex formation and activity are regulated by the cellular nutrient state. TORC1 inhibition strengthens the interaction of Atg1, Atg13 and Atg17 [33,75]; strongly enhances Atg1 kinase activity [33]; and therefore is required for the efficient formation of the PAS in bulk autophagy [40].

The Atg1 core complex member Atg13 is hyper-phosphorylated under nutrient-rich conditions, but becomes quickly de-phosphorylated upon TORC1 inactivation [33], making it a likely direct target of TORC1. Indeed, TORC1 can phosphorylate Atg13 in vitro [76]. Eight serine residues in Atg13 were identified as potential TORC1 phosphorylation sites, either by mass spectrometry or by sequence similarity to known TORC1 substrates. Alanine substitution of these residues blocked Atg13 phosphorylation as indicated by an increased mobility in SDS-PAGE. Overexpression of Atg13 constructs harboring different combinations of serine-to-alanine mutations, which mimic the non-phosphorylated state, was reported to induce bulk autophagy irrespective of TORC1 activity [76]. Whether these mutations also induce bulk autophagy at endogenous expression levels needs to be clarified. It is interesting to note that the overexpression of Atg13 constructs without a Ser496Ala mutation did not induce autophagy when TORC1 was active, even in the context of the seven other serine-to-alanine substitutions. The relevance of Atg13 Ser496 has been confirmed by work determining the crystal structure of the Atg13 regions interacting with Atg1 and Atg17 in complex with their respective binding partner [36]. The interaction of Atg1 with Atg13 was found to be mediated by tandem microtubule interacting and transport (TIMT) domains in the C-terminal domain of Atg1, which bind an MIT-interacting motif (MIM) in the central region of Atg13 (Fig. 2B). Intriguingly, the C-terminal half of the Atg13 MIM contains five serine residues found phosphorylated in mass spectrometry, among them Ser496 discussed above. Mimicking phosphorylation of these residues by mutation to aspartate severely reduced binding of Atg13 to Atg1, explaining the striking effect of Ser496 observed previously [36,76]. This leads to a model in which the N-terminal part of the Atg13 MIM mediates the constitutive binding of Atg1 and Atg13 reported previously [35], while phosphorylation of the C-terminal half regulates the binding affinity in response to the cellular nutrient state.

The same study [36] found the interaction of Atg13 and Atg17 to depend on hydrogen bonds between two serine residues in Atg13 (Ser428 and Ser429) and an aspartate residue in Atg17 (Asp427). Replacing Atg13 Ser429 with aspartate strongly impaired starvation-induced autophagy and the increase in Atg13–Atg17 binding usually observed under starvation. A serine-to-alanine substitution also showed a mild defect in autophagy and Atg17 binding, consistent with a functional role of the hydrogen bond formed by Ser429. Taken together, the data provide a mechanistic model for the formation of the yeast Atg1 core complex and its regulation by phosphorylation. While the involved serine residues are phosphorylated in a TORC1-dependent manner, it remains to be determined whether they are direct or indirect targets of TORC1.

As TORC1 activity inhibits Atg1 kinase activity, Atg1 might also be a substrate of TORC1. Several studies have identified phosphorylation sites on Atg2 [38,39,77–79], but assignment of kinases has remained sparse. This may in part be due to Atg1 autophosphorylation [31], which adds a level of complexity to phosphoproteomic studies. Five phosphorylation sites in Atg1 have been found to be significantly regulated by TORC1 activity, of which only one, Ser474, is downregulated upon rapamycin treatment [38]. This residue, however, is not conserved in other yeast species. The other four residues are conserved and their phosphorylation is upregulated upon TORC1 inhibition, indicative of Atg1-activating or autophosphorylation events. Indeed, Thr226 and an additional phosphorylation site, Ser230, are located in the kinase activation loop and required for Atg1 activity. Thr226 phosphorylation was identified and shown to depend on autophosphorylation and the core complex members Atg13 and Atg17 in an independent study [39]. Phosphorylation of Ser34 in the Atg1 N-terminus was proposed to repress Atg1 activity, as serine-to-aspartate or glutamate, but not serine-to-alanine substitutions at this position abrogated Atg1 kinase activity [77]. However, no kinase mediating this phosphorylation was identified, and no further investigation has followed up this finding. In summary, the potential direct action of TORC1 on Atg1 and its importance in autophagy regulation needs further investigation. It is conceivable that Atg1 activity is more indirectly regulated by TORC1-mediated changes in the binding affinity of other complex members toward Atg1. In line with this notion, autophosphorylation of Thr226 is thought to occur in trans, perhaps by dimerization of Atg1 mediated by Atg13 [37,38].

In higher eukaryotes, the Tor homologue mTOR (mechanistic target of rapamycin) in TORC1 also negatively regulates autophagy [80], and inhibition of TORC1 by starvation or rapamycin increases ULK1 kinase activity in mammalian cells [53,57]. Both ULK1 and Atg13 are phosphorylated in nutrient-rich conditions in vivo, but become de-phosphorylated upon starvation [54,56,57]. There is good evidence for a direct action of TORC1 on both proteins; TORC1 has been shown to phosphorylate both ULK1 and Atg13 in vitro [54,57]; in vivo, TORC1 overexpression increases Atg13 phosphorylation [56] and TORC1
activity is required for incorporation of radioactive $^{32}$P into Atg13 [57]; and ULK1 interacts with the TORC1 subunit Raptor (regulatory-associated protein of mTOR; Fig. 2b) [54,81]. However, similar to yeast, the identification of specific sites phosphorylated by TORC1 and their role in autophagy has remained a weak point. No data on TORC1-dependent phosphorylation sites are available for Atg13, and only two serine residues in ULK1 have been described as TORC1 targets, even though a multitude of phosphorylation sites in ULK1 have been found in mass spectrometry studies [82,83]. ULK1 Ser757 (residue numbering refers to mouse proteins unless stated otherwise) phosphorylation by TORC1 has been reported to regulate the interaction of ULK1 with AMP-activated protein kinase (AMPK) in two independent studies (Fig. 2b) [84,85]. In both studies, mutations of Ser757 to alanine and aspartate abrogated ULK1–AMPK1 binding, but the authors interpreted the data differently. Shang et al. concluded that TORC1-dependent phosphorylation was required for AMPK binding (implying that aspartate could not mimic phosphorylation), while Kim et al. argued that the serine residue itself was required for the interaction and phosphorylation would negatively regulate AMPK binding. Shang et al. also identified Ser637 as both a TORC1- and an AMPK-dependent phosphorylation site without a clear function in autophagy regulation.

While the exact role of TORC1 in ULK1 complex regulation remains to be elucidated, the results of several studies imply that TORC1 acts mechanistically different in mammals compared to yeast. As outlined above, yeast TORC1 regulates binding affinities within the Atg1 complex in response to nutrient cues. In contrast, the mammalian ULK1 core complex has been reported to change neither in composition nor in the binding affinities between its members when cells were starved or treated with rapamycin [53–57], and the Atg13–Atg101 and Atg13–ULK1 interactions within purified complexes persisted after treatment with phosphatase [65]. While subtle changes in complex affinities might have been masked by protein overexpression or overlooked, these findings indicate that TORC1 likely either regulates ULK1 kinase activity directly by phosphorylating yet to be identified ULK1 residues, or does so indirectly via effector proteins like AMBRA1 (activating molecule in BECN1-regulated autophagy protein 1). AMBRA1 has been shown to facilitate ULK1 ubiquitylation, increasing ULK1 stability and kinase activity. This is negatively regulated by TORC1-dependent phosphorylation of AMBRA1 Ser52 [86]. It is conceivable that TORC1 also regulates autophagy via routes not passing through the Atg1/ULK1 complexes in both yeast and higher eukaryotes. For instance, TORC1 regulates transcription in both yeast and mammals [74,87] and has been shown to regulate Atg14 expression in yeast [88].

Regulation by PKA

Akin to TORC1, the cAMP-dependent protein kinase A (PKA) is involved in the regulation of cellular metabolism and proliferation. In S. cerevisiae, several studies have found PKA to be a negative regulator of autophagy. PKA is activated by Ras proteins, and the expression of a constitutively active Ras2 mutant blocks autophagy induction by rapamycin treatment, while the expression of inactive Ras2 impedes proliferation and leads to autophagy induction [89]. Also, chemical inhibition of an ATP analogue-sensitive PKA mutant has been shown to induce autophagy [90]. In an evolutionary proteomics approach Atg1, Atg13 and Atg18 have been identified as potential PKA substrates [79]. PKA phosphorylates Atg1 at Ser508 and Ser515, and phosphorylation of these residues inhibits recruitment of Atg1 to the PAS, reproducing the phenotype of the constitutively active Ras2Val19 mutant. Intriguingly, serine-to-alanine substitutions of the Atg1 PKA sites rescue Atg1 PAS localization in the Ras2Val19 mutant, but not the induction of autophagy [79]. This implies the existence of other PKA targets in autophagy inhibition. In line with this, a later study by the same group reported phosphorylation of Atg13 by PKA on Ser344, Ser437 and Ser581 [91]. Similar to Atg1, Atg13 serine-to-alanine mutants showed increased localization to the PAS, even in the constitutively active Ras2Val19 background. Additionally, these mutations were found to increase the Atg13–Atg17 interaction. Ser437 lies adjacent to the Atg17-binding region in Atg13 (residues 424–436, Fig. 2b), but was not included in the Atg13 fragment crystallized with Atg17 [36]. Thus, a possible direct role of Atg13 Ser437 phosphorylation by PKA in Atg17 binding remains to be elucidated. It has been reported that the effects of TORC1 and PKA inhibition on autophagy induction are additive, suggesting that the two signaling cascades act independently [91].

In mammals, only one study has reported a link between PKA and autophagy [92]. Here, the regulatory PKA subunit Rla associates with structures positive for the isolation membrane marker LC3 (Microtubule-associated proteins 1 A/B light chain 3) and positively regulates autophagosome formation. As the same study found the PKA subunit Rla to also associate with mTOR and to negatively regulate mTOR activity, mammalian PKA likely acts indirectly in autophagy induction.

Regulation by Snf1/AMPK

AMPK is an important cellular energy sensor and regulates the transition from anabolic to catabolic metabolism upon energy stress. This role presents an obvious link between AMPK and autophagy. Interestingly and in contrast to PKA, more data are...
available on the role of AMPK in autophagy in higher eukaryotes than on the yeast AMPK homologue, sucrose non-fermenting 1 (Snf1).

Snf1 has been reported as a positive regulator of autophagy as Snf1 deficiency completely blocked autophagy induction [93]. In addition, the cyclin-dependent kinase Pho85 (phosphate metabolism 85) has been identified as a negative regulator of autophagy in the same study, but the mechanism of both Snf1 and Pho85 action in autophagy regulation remains to be elucidated.

In mammalian cells, AMPK has been found to transduce cytosolic calcium signals to induce autophagy [94] and to negatively regulate TORC1 activity by phosphorylation of TSC2 (tuberous sclerosis complex 2) and Raptor [95,96]. A direct link of AMPK and the ULK1 complex has been reported by several independent studies. Phosphorylation of Ser467, Ser555, Thr574 and Ser637 in ULK1 by AMPK was found to be required for p62/SQSTM1 (sequestosome-1) degradation during amino acid starvation, and for the autophagic degradation of mitochondria (mitophagy) [97]. In line with this, phosphorylation of ULK1 Ser555 by AMPK has recently been shown to be required for the translocation of ULK1 to mitochondria in hypoxia-induced mitophagy [98]. ULK1 translocation and mitophagy were found abrogated in AMPK-depleted cells, but could be restored by expression of a phosphorylation-mimetic ULK1 Ser555Asp mutant. Ser637 was reported as a major AMPK phosphorylation site on ULK1 in vitro [83], but the potential function of Ser637 phosphorylation in vivo has remained unclear. Two other sites on ULK1, Ser317 and Ser777, were also reported as direct AMPK targets [85]. Phosphorylation of these residues was found to be required for the induction of ULK1 activity and autophagy upon glucose withdrawal, but to be dispensable during amino acid deprivation. Interestingly, AMPK has been reported to interact with ULK1 (Fig. 2B) in several studies [83–85,99], but the data are ambiguous regarding the regulation of this interaction. The ULK1–AMPK1 interaction has been found to be negatively [84] or positively [85] regulated by TORC1 activity and TORC1-dependent phosphorylation of ULK1 Ser757. This may have been caused by different experimental conditions or because the ULK1–AMPK1 interaction changes over time during nutrient deprivation [83]. AMPK-dependent phosphorylation has been found to induce binding of 14–3–3 proteins to ULK1 [83,97,100], but the mechanistic significance of this interaction remains to be addressed.

Regulation of Atg1/ULK1 in selective autophagy

It is worth noting that studies aiming to elucidate the regulation of autophagy and Atg1/ULK1 kinase activity commonly have used global stimuli to induce autophagy, most of which drastically alter energy and nutrient availability within a cell or mimic this by directly activating or inhibiting central signaling hubs like TORC1. While these approaches are well suited to study events during general upregulation of autophagy, they fail to capture regulatory events in basal or locally induced selective autophagy. In mammals, a basal level of autophagy occurs also under non-stimulated conditions, highlighted by the presence of LC3-positive structures in fed cells (e.g., reported in Ref. [101]). This may be due to local activation of autophagy in a global inhibiting environment or due to incomplete basal inhibition of autophagy. In yeast, non-selective autophagy is completely repressed in nutrient-rich conditions [7,34], but the constitutively active Cvt pathway also requires Atg1 kinase activity [16,102]. First evidence on how Atg1 is activated during selective autophagy in the presence of global inhibitory signaling has recently been reported [103]. Atg1 activity in nutrient-rich conditions was found to depend on Ape1 complexes bound to the Cvt cargo receptor Atg19 and the selective autophagy scaffold protein Atg11. Approaches reconstituting Atg1 activity in vitro led to the conclusion that Atg19 and Atg11 only activated Atg1 in the presence of cargo complexes. Similar to the role of Atg19 and Atg11 in the Cvt pathway, Atg11 and the pexophagy cargo receptor Atg36 promoted Atg1 activity when peroxisomal damage was induced under nutrient-rich conditions. A model based on these findings suggests that Atg1 is activated when binding to selective autophagy cargo by clustering, inducing activation loop autophosphorylation in trans. An interesting question arising from this work is whether Atg1 binds to and is activated on cargo complexes only once they reach the PAS, or already in the cytosol. As Atg1 is not required for PAS recruitment of Ape1 complexes [104], the former is more likely, which would also help to restrict Atg1 activity to its site of action in a globally inhibitory setting. The study by Kamber et al. does not preclude additional regulatory steps and factors for two reasons. First, an Atg13 mutant specifically designed to mimic starvation conditions [76] was used in the in vitro experiments, which might obscure the requirement for regulatory elements by predisposing Atg1 for activation. Second, the experiments were carried out in yeast lysate, which may contain additional regulatory factors. For a profound understanding of Atg1 activation in selective autophagy, future studies need to address these possibilities. Another interesting direction for additional research is to elucidate whether similar mechanisms might govern selective autophagy in higher eukaryotes.

Autophagy-Related Substrates of Atg1/ULK1

Even though the pivotal role of Atg1/ULK1 in autophagy had long been established, the identification
of Atg1/ULK1 substrates has been slow until recently. This was likely caused by the lack of a published, well-defined consensus phosphorylation sequence of Atg1 and its homologues. We have recently deciphered the consensus motif of yeast Atg1 in its native complex [105]. We found Atg1 to have a unique phosphorylation motif, with strong selection for aromatic or aliphatic residues at the positions −3, +1 and +2 relative to the phosphorylation acceptor (Fig. 3a). As the Atg1 consensus motif matched well with a reported ULK1 target site in Beclin-1 [106], we reasoned that the Atg1 consensus motif needed to be highly conserved in ULK1. Indeed, the recently reported human ULK1 consensus [107] closely matches the yeast Atg1 consensus.

![Fig. 3. Atg1/ULK1 targets. (a) The consensus phosphorylation motif of *S. cerevisiae* Atg1. The logo was created using WebLogo 3.0 by aligning sites phosphorylated by Atg1[105,127]. (b) The consensus phosphorylation motif of *H. sapiens* ULK1. The logo was created using WebLogo 3.0 from positional scanning peptide library data [107,127]. (c) Alignment of Atg1 and ULK1 phosphorylation sites in yeast, fly and mammals. Underscore: phenotype associated with this site reported; gray text: low-matching sequence and/or conflicting data—see text for details; italic: unreported target site with matching sequence and/or homology to a reported target. The defining −3, +1 and +2 positions of the Atg1/ULK1 consensus are highlighted in gray, and preferred residues are color coded at these positions. Red: Met, Leu, preferred at −3; green: Ile, Val, Phe, Tyr, Trp preferred at +1 and +2; blue: Ser preferred at +1 and +2 (ULK1 only). (d) Alignment of the potential Atg1/ULK1 target site in fly (*D.m.*). Paxillin with its zebrafish (*D.r.*), mouse (*M.m.*), rat (*R.m.*), and human (*H.s.*). Framed: consensus motif; color code as in (c). (e) Alignment of the reported ULK1 target sites in rat (*R.n.*) AMPKγ1 with its human (*H.s.*) and yeast (*S.c.*) homologues. Framed: consensus motif; color code as in (c).}
consensus in its selection for aromatic or aliphatic residues at positions −3, +1 and +2 from the phosphorylation acceptor site, but differs in some details: compared to Atg1, ULK1 has stronger specificity for Met at position −3, prefers aromatic over aliphatic residues at position +1 and does not select against Ser at position +2 (Fig. 3b). Interestingly, in contrast to the ULK1 peptide consensus, ULK1 in vivo targets are not enriched in hydrophobic residues at position +2 [107]. We found no such striking differences between the Atg1 peptide consensus and in vivo phosphorylation sites in Atg9 [105]. Both Atg1 and ULK1 strongly prefer serine over threonine as phosphorylation acceptor. This is in line with a conserved Phe residue immediately following the Asp-Phe-Gly (DFG) motif in the kinase domain, which has been shown to confer serine specificity in serine/threonine kinases [108]. The deciphering of the Atg1 and ULK1 consensus motifs has already led to the identification of novel substrates of these kinases and will greatly facilitate future screens for putative targets of Atg1/ULK1. Such screens will have to be followed up by thorough characterization of the identified potential target sites in vivo, as phosphorylation events do depend not only on consensus motifs but also on the accessibility of the phosphorylation site and the localization of the substrate and the kinase in the cell. Nevertheless, knowledge of the Atg1/ULK1 phosphorylation motifs allows for critical evaluation of potential and published Atg1/ULK1 substrates, which we will endeavor in the following sections.

Atg1 substrates in D. melanogaster

In D. melanogaster, Atg1 has been reported to phosphorylate paxillin (dPax) [109] and spaghetti-squash activator (Sqa) [110]. A genetic interaction of the cytoskeletal scaffolding protein paxillin and Atg1 governs wing morphology and arista patterning, and fly Atg1 directly phosphorylates an N-terminal fragment of dPax comprising residues 1–346 in vitro [109]. Similar to fly Atg1, mouse ULK2 phosphorylates an N-terminal fragment of murine paxillin. Paxillin has been found to be required for autophagy in fly and mammalian cells [109], but neither the role of paxillin phosphorylation nor the precise site of phosphorylation has been elucidated to date. Using ScanSite [111] and the yeast Atg1 consensus motif [105], we found dPax Ser260 to be a well-scoring Atg1 consensus site (Fig. 3c). The consensus motif is highly conserved in mouse and zebrafish paxillin (Ser272 and Ser248, respectively, Fig. 3D), implying functional importance and making this site an interesting target for future studies.

The kinase domain of the D. melanogaster myosin light chain kinase Sqa is phosphorylated by Atg1 in vitro, and Thr279 has been reported as the main phosphorylation site [110]. A threonine-to-alanine substitution at this position has been found to impair Sqa phosphorylation in vitro and myosin II activation and GFP-Atg8a puncta formation in vivo. While the data clearly link Atg1 to Sqa and myosin II activation, Thr279 seems to be an unlikely Atg1 target site for several reasons. First, the sequence flanking Thr279 does not conform well to the Atg1 and ULK1 consensus motifs (Fig. 3c). Second, D. melanogaster Atg1, like its yeast and mammalian homologues, features a phenylalanine residue at the DFG +1 position. This confers specificity for serine as a phosphorylation acceptor [108], making threonine an improbable substrate residue. Third, the corresponding residue in the mammalian homologue DAPK3/ZIPK (death-associated protein kinase 3/zipper-interacting protein kinase), Thr265, has been reported as a major autophosphorylation site [112,113]. Thus, phosphorylation by Atg1 may trigger Sqa autophosphorylation in D. melanogaster, but Sqa Thr279 does not seem to be a likely target residue of Atg1.

Atg1 substrates in S. cerevisiae

Even though the yeast S. cerevisiae has been a highly prominent model organism in autophagy research, Atg1 substrates have remained elusive for more than a decade. We recently reported the first Atg1 substrates in budding yeast, Atg2 and Atg9 [105]. Phosphorylation of both proteins by Atg1 has been confirmed in a recent study [103]. We found Atg1 to phosphorylate Atg2 at Ser249 and Atg9 at six residues, Ser19, Ser657, Ser802, Ser831, Ser948 and Ser969 (Fig. 3c). Atg2 mutants harboring serine-to-alanine substitutions at Ser249 and Ser1086, which also matches the Atg1 consensus motif, did not elicit a phenotype in starvation-induced autophagy or the Cvt pathway, indicating either functional redundancy or a specialized role in another type of selective autophagy. In contrast, our data show phosphorylation of Atg9 to be a crucial requirement for both Cvt pathway and bulk autophagy progression. In line with findings reported previously [114], we found that Atg9 phosphorylation facilitates binding of Atg18 to Atg9. Atg9 phosphorylation is essential at an early stage of autophagosomal formation: it is required for the stable recruitment of Atg18 to the PAS, and the formation and expansion of the isolation membrane [105]. Interestingly, the serine-to-alanine substitutions at the Atg1 phosphorylation sites did not change Atg9 localization, while a deletion of Atg1 leads to Atg9 accumulation at the PAS [114]. This indicates that Atg1 regulates Atg9 shuttling by other means than Atg9 phosphorylation. In line with a slightly different role of mammalian Atg9 in autophagy [115], sequence homology of yeast and mammalian Atg9 is low in the cytosolic termini, and the yeast phosphorylation sites are not conserved in mammals. Whether ULK1 phosphorylates mammalian Atg9 is to be determined in future studies.

Very recently, yeast Atg6 has been reported to be phosphorylated by Atg1 [103], similar to its mammalian
homologue Beclin-1, which is a substrate of ULK1 [106,107]. However, no specific phosphorylation sites have been identified. The major ULK1 phosphorylation site in mouse Beclin-1, Ser14 [106,107], seems not to be conserved in Atg6. Human Beclin-1 Ser279 and Ser337, reported ULK1 target sites [107], are homologous to Atg6 Ser332 and Ser390, respectively. Both sites fit the Atg1 consensus moderately well (Fig. 3c). Two serine residues on yeast Atg6, Ser85 and Ser40, are exceptionally good matches for the Atg1 phosphorylation motif (Fig. 3c). Atg6 Ser85 is conserved in S. pombe, but not in Candida albicans. S. cerevisiae Atg6 Ser540 is conserved in S. pombe Atg6 Ser462 and corresponds to the negatively charged Glu514 in C. albicans Atg6, hinting at a potential role of a negative charge at this position. However, Ser462 is the antepenultimate residue in S. pombe Atg6; whether Atg1 can phosphorylate residues this close to the C-terminus has not been determined yet.

**Mammalian ULK1 substrates**

In mammals, ULK1 has been found to phosphorylate its binding partners Atg13, FIP200 and Atg101 [107,116]. In a SILAC (stable isotope labelling by amino acids in cell culture) mass spectrometry experiment, ULK1 was found to phosphorylate human Atg13 isoform 2 at Ser318 in a manner dependent on the Hsp90–Cdc37 (heat shock protein 90–cell division cycle 37) chaperone complex [116]. This residue corresponds to Ser354 and Ser355 in the canonical murine and human isoforms, respectively. Atg13 Ser355 phosphorylation is required for targeting Atg13 to depolarised mitochondria and for mitophagy, but not for starvation-induced autophagy. This indicates that ULK1 might target different substrates and phosphorylation sites depending on the cue that triggers autophagy activation. Human Atg13 S355 passably conforms to the ULK1 consensus motif (Fig. 3c), but could not be confirmed as an ULK1 phosphorylation site in another study by Egan et al. [107], possibly due to weak coverage of this region in mass spectrometry. Egan et al. reported human Atg13 Ser389 to be a target of ULK1 (Fig. 3c), but did not characterize it regarding a potential function in autophagy. Given the close interaction of ULK1 and Atg13, and the fact that Atg13 has been suggested to be a substrate of ULK1 already when it was identified [54,57–59], clarification of Atg13 phosphorylation by ULK1 will be an interesting subject for future studies. The same holds true for FIP200, which has been found to be phosphorylated depending on ULK1 activity already in an early study [57]. Evidence for phosphorylation of three FIP200 sites by ULK1 has been provided [107], but their functional significance has not been assessed. The reported sites in human FIP200 are Ser943, Ser986 and Ser1323, of which only Ser1323 is conserved in murine FIP200 (Fig. 3c). This indicates that Ser943 and Ser986 phosphorylation might rather play a species-specific role in Homo sapiens. Two ULK1 phosphorylation sites were identified in human Atg101: Ser11 and Ser203 [107]. Both sites match the ULK1 consensus well (Fig. 3c) and are conserved in mouse and zebrafish. Similar to FIP200, evidence for a role of Atg101 phosphorylation in autophagy is lacking.

ULK1 has also been reported to phosphorylate several members of the autophagy-specific PI3KC3 complex 1: Beclin-1, Vps34/PIK3C3 (vacuolar protein sorting 34/phosphatidylinositol 3-kinase catalytic subunit type 3), and AMBRA1 [106,107,117]. The PI3KC3 complex 1 plays a central role in autophagy by phosphorylating phosphatidylinositol to generate PI3P, allowing for the recruitment of PI3P-binding proteins like DFCP1 and members of the WIPI family [118]. ULK1 has been reported to phosphorylate AMBRA1 in vivo and in vitro, and to be required for the abrogation of PI3KC3 complex 1 binding to the dynein motor complex upon starvation [117]. This binding is mediated by AMBRA1, and the overexpression of AMBRA1 mutants unable to bind to the dynein motor complex upregulates autophagy in fed cells. This implies that ULK1-mediated phosphorylation of AMBRA1 is a key step in autophagy induction. Two ULK1 phosphorylation sites in human AMBRA1 have been reported, but not characterized further: Ser465 and Ser635 (Fig. 3c) [107]. Both sites are conserved in mouse AMBRA1, and Ser465 also in the zebrafish homologue. In mouse, ULK1 has been found to activate the PI3KC3 complex 1 by directly phosphorylating Beclin-1 Ser14 (Fig. 3c), and this phosphorylation event is required for autophagy induction upon nutrient deprivation [106]. Phosphorylation of this residue has been confirmed in human Beclin-1 [107], among several other ULK1-dependent phosphorylation sites. These sites include Ser279 and Ser337 (Fig. 3c), both of which are conserved in yeast. Human Vps34, the catalytic subunit of the PI3KC3 complex 1, is also phosphorylated by ULK1 at Ser249 (Fig. 3c) [107]. While this residue is conserved in yeast, a serine-to-alanine substitution did not elicit a phenotype in starvation-induced autophagy. As in yeast Atg2 [105], this might be due to a redundant or highly specialized function of Vps34 Ser249 phosphorylation. In summary, there is striking evidence for the regulation of the PI3KC3 complex 1 by ULK1, but future studies will need to determine the functional role of several reported phosphorylation events.

FUNDC1 is an outer-membrane protein that acts as a cargo receptor protein in hypoxia-induced mitophagy [119]. Phosphorylation of mouse FUNDC1 Ser17 by ULK1 is required for efficient binding of LC3 to FUNDC1 and mitophagy progression [120]. Mouse FUNDC1 Ser17 matches the ULK1 consensus (Fig. 3c) and is conserved in human and zebrafish, lending additional support to its role as a bona fide ULK1 target site. Strikingly, expression of a
serine-to-aspartate mutant mimicking phosphorylation was sufficient to drive mitophagy even in the absence of ULK1 [120]. This might hint that only ULK1, but not ULK2 phosphorylates FUNDC1, while the two kinases might act redundantly in other steps of mitophagy.

Atg1/ULK1-driven feedback loops

In several studies in D. melanogaster and mammalian cells, Atg1 and ULK1 have been implicated in negative regulation of TORC1 activity toward two major substrates, S6K (ribosomal protein S6 kinase) and 4E-BP1 (Eukaryotic translation initiation factor 4E-binding protein 1) [57,58,81,121,122]. It is tempting to think of TORC1 as a direct target of Atg1 or ULK1, forming a positive feedback loop in autophagy induction. Indeed, ULK1 has been found to phosphorylate the TORC1-specific subunit Raptor [81], and five human Raptor residues have been reported as ULK1 targets: Ser792, Ser855, Ser859, Ser863 and Ser877 (Fig. 3C) [122]. Ser792 matches the ULK1 phosphorylation motif nicely, but has previously been reported as an AMPK target site [95]. Ser855 and Ser859 conform to the ULK1 consensus motif reasonably well and are the most likely targets of ULK1 among the reported sites, as neither Ser863 nor Ser877 matches the ULK1 phosphorylation motif. Strikingly, Ser863 is a reported target of mTor [123]. The upregulation of phosphorylation at an mTOR target site in Raptor and an autophosphorylation site in mTOR by ULK1 overexpression indicate that ULK1 does not repress the catalytic activity of TORC1 per se [122]. TORC1-activating cues have been shown to induce conformational changes in TORC1, weakening the binding of Raptor to the complex [123,124]. Conversely, both ULK1 and ULK2 have been reported to stabilise Raptor in TORC1, implying that they might regulate mTOR kinase domain accessibility rather than activity [81]. Whether this is achieved by phosphorylation of Raptor Ser855 and Ser859 remains to be determined.

ULK1 has been found to phosphorylate AMPK and to negatively regulate AMPK activity, constituting a negative feedback loop which attenuates autophagy-inducing signaling [99]. ULK1 has been reported to phosphorylate AMPK in vitro at three sites in the AMPKα1, four sites in AMPKβ2 and two sites in AMPKγ1 (Fig. 3C). However, no direct evidence for a functional role of these sites has been provided, and for several of them, direct targeting by ULK1 is hard to conceive. These include threonine residues and serine residues in an unfavourable sequence context, most of which are not conserved in either D. melanogaster or S. cerevisiae. Ser260 and Ser269 in rat AMPKγ1 might be the most promising targets, as they are conserved and correspond well to the ULK1 consensus motif in human AMPKγ1, and are present also in yeast Snf4, albeit flanked by sequences matching the Atg1 phosphorylation motif only moderately (Fig. 3c and e).

Conclusion

Since the identification of TORC1 as a major regulator of yeast autophagy in 1998, our understanding of the regulatory network governing autophagy repression and induction has greatly advanced, both in terms of underlying signaling cascades and their targets (Fig. 4). TORC1 and PKA are the major repressors of autophagy in non-challenged cells, whereas AMPK triggers autophagy induction in response to several cues, including nutrient stress. These pathways have been shown to regulate, often directly, Atg1/ULK1 activity, making Atg1/ULK1 the pivot of autophagy signaling. Recently, an important piece to this puzzle has been added by discovering a potential mechanism for yeast Atg1 activation in selective autophagy, when global signaling inhibits the induction of non-selective autophagy. It will be interesting to see whether a similar mechanism exists in higher eukaryotes. While the big picture of autophagy regulation has advanced in great steps, the understanding of the mechanistic events is largely lacking. Future studies should aim to elucidate the mechanisms by which phosphorylation events or other post-translational modifications exert their function, and how the identified factors cooperate in autophagy regulation. How does phosphorylation by AMPK increase ULK1 activity? What molecular changes are induced in ULK1 upon phosphorylation by TORC1, rendering it inactive? Comprehensive and rigorous biochemical analyses together with structural approaches will help to answer such questions.

Progress in identifying downstream effectors of Atg1/ULK1 has been slow in the beginning, but has picked up the pace over the last few years. Today, a range of Atg1/ULK1 targets in several organisms are known (Fig. 3C), including several in not autophagy-related pathways [125,126], which we did not cover in this review. In line with its role as an autophagy signaling hub, Atg1/ULK1 acts at many levels of autophagy, phosphorylating cytoskeletal organizers and regulators of motor proteins, cargo receptors, other kinase complexes and core autophagy proteins in different organisms (Fig. 4; see the appropriate sections for details). Knowledge of the Atg1 and ULK1 consensus motifs will facilitate the discovery of additional Atg1/ULK1 substrates and regulatory pathways downstream of these kinases. Similar to studies focusing on the regulation upstream of Atg1/ULK1, future studies on downstream effectors should not only identify Atg1/ULK1 target sites but also characterize their mechanistic role in the progression of autophagy. Asking “how?” more often than “what?” when studying potential targets of this central autophagy kinase will allow deciphering the mechanistic events underlying autophagy signaling.

In addition to further clarifying the mechanistic roles of phosphorylation in Atg1/ULK1 regulation and
function, the identification of counteracting protein phosphatases should be an important aim of future autophagy research. Knowledge on protein phosphatases involved in autophagy pathways is very limited. This is in stark contrast to their likely important role, especially considering the rapid dephosphorylation events observed upon autophagy induction in yeast.

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- Autophagy
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**Fig. 4.** Regulation of autophagy by signaling through the Atg1/ULK1 complex. The activity of Atg1 and ULK1 is directly or indirectly regulated by TORC1, AMPK and PKA. In yeast, Atg1 phosphorylates Atg9 to promote PAS organization and isolation membrane formation. Atg1 also targets Atg2 and the PI3K complex member Atg6, but the role of these phosphorylation events has not yet been elucidated. In mammals, ULK1 phosphorylates its complex partners Atg13, Atg101 and FIP200. Phosphorylation of Atg13 and the cargo receptor protein FUNDC1 is required for mitophagy. ULK1 activates the PI3KC3 complex 1 by phosphorylating Beclin-1 and AMBRA1; the role of Vps34 phosphorylation has remained unclear. ULK1 also phosphorylates the TORC1 component Raptor and several AMPK subunits and thus regulates the activity of the upstream signaling complexes. Note that crosstalk between TORC1, AMPK and PKA exists, but is not shown in this model.
Abbreviations used:
CMA, chaperone-mediated autophagy; PAS, phagophore assembly site; Ape1, aminopeptidase 1; Cvt, cytosol-to-vacuole targeting; HORMA, Hop1, Rev7 and Mad2; PI3P, phosphatidylinositol 3-phosphate; PI3KC3, phosphatidylinositol 3-kinase class 3; MIM, MIT-interacting motif; AMPK, AMP-activated protein kinase; PKA, protein kinase A; TORC1/2, Target of rapamycin complex 1/2.

References
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